Assessing the Use of Local Indigenous Microorganisms for the Bioremediation of Sites Contaminated With Petroleum Hydrocarbons

BY

Feras Mohammad Salamah

A Thesis Presented to the

DEANSHIP OF GRADUATE STUDIES

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN, SAUDI ARABIA

In Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

In

Environmental Sciences

May, 2011

KING FAHD UNIVERSITY OF PETROLEUM AND MINERALS

DHAHRAN 31261 .SAUDI ARABIA

DEANSHIP OF GRADUATE STUDIES

This thesis, written by Feras Mohammad Rasheed Salamah under the direction of his thesis advisor and approved by his thesis committee, has been presented and accepted by the Dean of Graduate Studies in partial fulfillment for the Degree of MASTER OF SCIENCE IN ENVIRONMEMTAL SCIENCES

THESIS COMMITTEE

Dr. Amjad Khalil
(Chairman)

Dr. Bassam Tawabini
(Member)

Dr. Alaadin Bukhari
(Member)

Dr. Abdul Aziz M. Al-Shaibani
Department Chairman

Dr. Salam Zummo
Dean of Graduate Studies

Date 7/6/11

II

DEDICATION

THIS THESIS IS GRATEFULLY

DEDICATED

TO

THE

Future of Our Nation

My Parents

My Wife

&

My Children

ACKNOWLEDGMENTS

I sincerely express my gratitude and thanks to Allah for giving me this opportunity to explore the knowledge through King Fahd University for Petroleum and Minerals (KFUPM), and I shall always remember Allah with full gratitude.

I would like to express my deep appreciation to Dr. Amjad Khalil, Biotechnology research group, physics department, the chairman of my thesis committee, for his guidance and support throughout my work on this thesis. I also would like to thank Dr. Bassam Tawabini, the member in thesis committee, and my academic adviser for his successful supervision throughout the study. Special thanks to Dr. Aladdin Bukhari, the member in thesis committee for his efforts and valuable reviewing suggestions are highly appreciated.

I acknowledge the kind support and cooperation of Dr. Abdul Aziz Al-Shaibani, Chairman of Earth Sciences, KFUPM, and his staff and secretary. I take this opportunity to thank Dr. Magdi El-Said who is specialist in biodegradation in ARAMCO, for his advices, suggestions and exchange of ideas. In addition, I also appreciate the valuable support of my colleagues and friends. Hence it is my pleasure to thank Mr. Hassan Abd EL-Rahman (specialist in biology), Dr. Nemer Mehana (specialist in analytical chemistry), my manager Mr. Ahmed AL-Retoei and others.

Last but not the least; my grateful thanks to King Fahd University of Petroleum and Minerals (KFUPM) for giving me this opportunity to complete my Master degree in the field of environmental sciences...

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THESIS ABSTRACT

Name: Feras Mohammad Salamah

Title: Assessing the Use of Local Indigenous Microorganisms for the Bioremediation of Sites Contaminated With Petroleum Hydrocarbons

Major Field: Environmental Sciences

Date of Degree: may, 2011

The main objective of this study was to investigate the isolation and characterization of bacterial isolates from Aramco contaminated sandy soil site with crude oil. Two strains were isolated and characterized by sequencing the 16S rRNA gene, these strains are Psuedmonasxanthomonad sp. and Bacillus sp.

The removal of total petroleum hydrocarbon using consortium of the two strains was investigated under 3 environmental factors, temperature, pH, and nutrient concentrations.

The effect of nutrients on the removal of TPH was investigated using 10mM of Ammonium chloride, 10 mM Sodium nitrate and 10 mM Urea. Ammonium chloride show that it is the best nitrogen source for removal of TPH by bringing down the TPH to 44.5% followed by Urea 48% and Sodium nitrate 49.5%.

The effect of three different concentrations of ammonium chloride on the removal of TPH was also investigated; these concentrations are 5, 10, and 20 mM. At 5 mM Ammonium chloride, TPH was gone down to 30% followed by 10 mM (48%) and 20 mM (77.5%).

The effect of pH (5, 7, and 9) on the removal of TPH was investigated under 5mM ammonium chloride. At pH 7.0 the consortium of bacteria was the most efficient (TPH down to 55%).

The effect of temperature on the removal of TPH was also investigated. Temperatures used 25, 35, and 45 ^{0}C. The best removal efficiency of TPH was at 35 ^{0}C (TPH down to 45%), at 25 ^{0}C the removal went down to 57% followed by 45 ^{0}C (TPH down to 72%).

ملخص الرسالة

الاسم : فراس محمد رشيد سلامة

عنوان الرسالة :

تقييم استخدام الأحياء الدقيقة المستوطنة في المعالجة الحيوية للمواقع الملوثة بالشتقات البترولية الهيدركربونية

التخصص :علوم البيئة

تاريخ التخرج :١٤٣٢ هـ / ٢٠١١ م

تتلخص هذه الدراسة في عزل كائنات حية دقيقة مستوطنة (بكتيريا) من تربة رملية ملوثة بالمواد الهيدروكربونية (المشتقات البترولية) من موقع نفطي يتبع شركة أرامكو السعودية في جدة، حيث تم عزل نوعين من البكتيريا، وتم تعريفها جينيا عن طريق الحمض النووي ، وتبين أنهما (نوع من الزيدوزانثوموناس) و(نوع من الباسيليس).

تم بعدها تقييم أثر وفاعلية خليط من هذين النوعيين من البكتيريا على التحلل الحيوي للمواد الهيدروكربونية الموجودة في تربة ملوثة بالمواد النفطية. وتم دراسة ثلاثة عوامل بيئية وهي (المواد المغذية وتراكيزها ودرجة الحموضة ودرجة الحرارة) لاختيار أفضل العوامل البيئية المؤدية إلى أكبر قدر من التحلل الحيوي للمواد الهيدروكربونية.

تم تقييم أثر المواد المغذية على فاعلية التحلل الحيوي للبكتيريا باختيار ثلاثة مصادر نيتروجينية وهي (كلوريد الأمونيوم ونترات الصوديوم واليوريا وبتركيز 10 mM لكل منها) ،باعتبار أن النيتروجين من أهم المصادر الغذائية للبكتيريا. تبين بأن المصادر الغذائية المختارة كانت متقاربة في نتائجها على أفضلية لكلوريد الأمونيوم والذي خفض نسبة المواد الهيدروكربونية في عشرة جرامات من التربة خلال عشرين يوماً إلى 44.5% تلاه اليوريا بنسبة ٤٨% وآخرها نترات الصوديوم بنسبة 49.5%.

كذلك تم تقييم أثر تركيز المواد المغذية على فاعلية البكتيريا باختيار ثلاثة تراكيز لكلوريد الأمونيوم وهي (٥ و١٠ و٢٠ ميلي مولار). تبين بأن أفضل تركيز كان عند تركيز ٥ ميلي مولار حيث انخفضت كمية المواد الهيدروكربونية في عشرة جرامات إلى ٣٠% ثم عند التركيز ١٠ ميلي مولار والذي أخفض نفس الكمية إلى نسبة ٤٨% ، بينما كان أثر الزيادة سلبيا عند ٢٠ ميلي مولار حيث نزلت الكمية نفسها إلى نسبة ٧٧.٥% فقط.

تم اختيار كلوريد الأمونيوم كمصدر للنيتروجين في تقييم أثر درجة الحموضة على فاعلية البكتيريا في التحلل الحيوي للمواد الهيدروكربونية حيث تم اختيار ثلاث قيم وهي (٧ و٥ و٩). تبين بأن فاعلية البكتيريا في التحلل الحيوي كانت الأفضل عند درجة الحموضة (٧) حيث خفضت كمية المواد الهيدروكربونية في عشرة جرامات إلى 55% بينما كانت القيم متقاربة عند درجة الحموضة ٩ ودرجة الحموضة ٥ حيث انخفضت كمية المواد الهيدروكربونية إلى ٨١% و إلى ٧٩.٥%على الترتيب.

كذلك تم تقييم أثر درجة الحرارة على فاعلية البكتيريا في التحلل الحيوي للمواد الهيدروكربونية باختيار ثلاث درجات وهي (٢٥ و٣٥ و٤٥ درجة مئوية). كانت فاعلية البكتيريا الأفضل على حرارة ٣٥ درجة مئوية حيث انخفضت كمية المواد الهيدروكربونية في عشرة جرامات إلى ٤٥% تلتها درجة حرارة ٢٥ مئوية حيث انخفضت الكمية نفسها إلى نسبة ٥٧% ثم حرارة ٤٥ مئوية حيث كان أثرها ضعيفاً حيث نزلت الكمية إلى نسبة ٧٢%. مع اعتبار انخفاض بسيط في كمية المواد الهيدروكربونية في التجارب الضابطة السابقة كلها لعوامل غير حيوية.

CHAPTER ONE

1.1. INTRODUCTION:

Saudi Arabia is the largest producer of petroleum and its related products such as gasoline, diesel, or fuel oil in the world. Oil companies are responsible for looking after these activities through its management of oil fields, off-shore drilling structures, transportation and refining, and major crude and refined oil storage facilities. These activities, spread over the period of the last 60 years, might have resulted in soil and ground water contamination through leaks, spills, accident, etc. and can become a source of serious damages to the environment in future. Experiences with this type of contamination in the USA, Europe and Japan are serious warnings for environmentally conscious organizations dealing with such activities. Several studies have shown that exposure to petroleum products resulting from contaminated soils may occur via the following routes; inhalation, dermal absorption, consumption of plants and animals that have assimilated petroleum products, and by consumption of contaminated drinking water [1].

The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry [2]. In addition, bioremediation technology is believed to be noninvasive and relatively cost-effective [3]. Biodegradation by natural populations of

Microorganisms represent one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment [4] and is cheaper than other remediation technologies [5].

The success of oil spill bioremediation depends on one's ability to establish and maintain conditions that favor enhanced oil biodegradation rates in the contaminated environment. Numerous scientific review articles have covered various factors that influence the rate of oil biodegradation [6-11]. One important requirement is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is between 6 and 9. The physical and chemical characteristics of the oil and oil surface area are also important determinants of bioremediation success. There are the two main approaches to oil spill bioremediation: (a) bioaugmentation, in which known oil-degrading bacteria are added to supplement the existing microbial population, and (b) biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting cosubstrates.

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Petroleum hydrocarbons can be divided into four classes: saturates, the aromatics, the asphaltenes (phenols, fatty acids,

ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) [12]. Different factors influencing hydrocarbon degradation have

Been reported by Cooney et al. [13]. One of the important factors that limitbiodegradation of oil pollutants in the environment is their limited availability to microorganisms. Petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded [14]. Hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkane's, branched alkanes, small aromatics cyclic alkanes [15]. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all [16].

Microbial degradation is the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon pollutants from the environment [17-19]. The recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Jones et al. [20]. They studied the extensive biodegradation of alkyl aromatics in marine sediments which occurred prior to detectable biodegradation of n-alkane profile of the crude oil and the microorganisms, namely, *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus* were found to be involved for alkylaromatic degradation. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye et al. [21]. Nine bacterial strains, namely, *Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus* sp*., Alcaligenes*sp., *Acinetobacterlwoffi, Flavobacterium sp., Micrococcus roseus, and Corynebacterium*sp. Were isolated from the polluted stream which could degrade crude oil.

Bacteria, fungi, and other microorganisms are found in vast numbers in most soils and significant numbers persist even at depths of several hundred feet. These microorganisms can adapt to and metabolize a wide range of petroleum hydrocarbons [22]. Oil-spill bioremediation methods aim at providing favorable conditions of oxygen, temperature and nutrients to maximize biological hydrocarbon breakdown. Such methods have been applied successfully in restoring polluted seashores, airports, military operations, power plants, etc. [23].

This study investigated the isolation and characterization of indigenous microorganisms capable of degrading total petroleum hydrocarbons from contaminated sites. Their potential of enhanced in situ biodegradation treatment of petroleum hydrocarbon contaminated soils was also investigated under different conditions of temperatures, pH, and nutrients.

1.2.OBJECTIVES :

The main objective of this study is to investigate the potential of enhanced in situ biodegradation treatment of petroleum hydrocarbon contaminated soils. The specific objectives include the following**:**

1. Isolation and characterization of indigenous microorganisms capable of degrading total petroleum hydrocarbons from contaminated sites.

2. Evaluate the potential of enhanced biodegradation in the removal of total petroleum hydrocarbons (TPH) from contaminated sites. This will be conducted under different conditions of temperatures, pH, nutrients and time.
3. Identify the optimum bio-treatment conditions of hydrocarbons contaminated soil.

1.3. DESCRIPTION OF THE PROBLEM:

Bioremediation processes are biological treatment processes that improve or stimulate the metabolic capabilities of microbial population to degrade organic compounds. However, under certain adverse non optimum field conditions, organic compounds may persist for a long time. The factors that may prevent microbial degradation include the following:

- Chemical concentration that are toxic to micro-organisms
- Inadequate type or low number of micro-organisms due to toxic conditions
- Lack of nutrients such as nitrogen, phosphorous, potassium, sulphur, or trace elements (most petroleum wastes are not nutritionally balanced)
- Unfavorable moisture conditions
- Lack of oxygen or other electron acceptors

Therefore, it is very important to understand such conditions so that biological process can be applied successfully.

The major outcome of this thesis is developing sufficient information about in situ biological treatment process, which will allow us to utilize natural and less expensive technology in the cleanup of the major contaminated sites. Furthermore, the data collected can be used in the development of a model for the cleanup of the contaminated sites. The in-situ biotreatment method is an innovative technology and has been recommended for the treatment of many contaminated sites [24, 25].

Bioremediation is a process by which chemical substances are degraded by bacteria and other microorganisms. The use of these microorganisms has been successfully applied for the treatment of waste and wastewater in controlled systems [26, 27]. Several research studies have been recently performed to investigate the use of bioremediation for oil-spill cleanup in seawater, freshwater and terrestrial areas. The technique has been found to have a potential for broad applications in terrestrial and freshwater environments for treating soils and sediments contaminated with oil and other substances, as well as for coastal environments impacted by oil spills. Water is a more sensitive medium than soil and requires different remediation techniques. Spills to surface water are easier to clean up than spills to groundwater, for obvious reasons. It is not only much harder to see the extent of the contamination, but also to remove the source of the contamination as, for example, a leaking underground storage tank.

1.4. APPROACH TO THE PROBLEM:

The approach to the project was experimental and resulted in isolating and enriching microbial strains from the examined contaminated sites. Numbers of bench-scale experiments were carried out in order to assess the biodegradation enhancement process. The assessment of the biodegradation was achieved by determination of total petroleum hydrocarbons (TPH) by gravimetric methods. The approach followed in this study summarized as follow:

- Isolating and enriching microbial strains from the examined contaminated soil in respect to physical, chemical and biological parameters.
- Carry out a number of laboratory bench-scale experiments in order to assess the biodegradation enhancement process and identify the optimum biotreatment conditions of nutrient, pH, and temperature.

CHAPTER TWO

2.1. LITERATURE SURVEY

Ghulam Shabir et al. [28] investigated the biodegradation of kerosene in soil using a mixed bacterial culture under two levels of nutrients: (C1) low nutrient concentration as compared to (C2), both of them contained 4 % (w/w) kerosene in soil as a sole carbon source. After 6 weeks of incubation: (C1) and (C2) exhibited 27±3% and 65±7% kerosene degradation, respectively. The highest bacterial growth was observed in (C2) with a significant reduction in nutrient content of soil over2–3 weeks of incubation. Overall, 46±12% and 54±24% of nitrogen, 36±3% and 43±3% of phosphorus and 24±2% and 35±2% of potassium content of the soil were depleted under (C1) and (C2) respectively. Briefly, their work has defined nutrient requirements for kerosene oil degradation and its remediation from contaminated soil.

Jiin-Shuh Jean et al. [29] demonstrated that the morphological adaptation of Pseudomonas spp. is strongly influenced by temperature and nutrient levels in an environment. They studied the effects of inorganic nutrient (sulfate, phosphate, and ammonium chloride) levels on the aerobic biodegradation of benzene, toluene, and xylene (BTX) by Pseudomonas spp.in a laboratory using a glass sand tank. They found that the increase of nutrient levels resulted in enhanced bacterial growth and BTX degradation. Sulfate and phosphate serve as electron acceptors in the microbiological Processes degrading BTX. Also, they assessed the influence of the different environmental parameters (temperature, nutrient levels, and incubation time) on

microbial morphology. They noticed that bacterial morphological changes during BTX degradation reveal that the filamentous bacteria were the dominant species at low temperatures about (20c) while the spherical and rod-shaped cells became dominant at higher temperatures ranging from (25-28 c) and pH ranging from (7–7.5). The morphological adaptation appears to be controlled by the temperature and nutrient levels in the sandy medium where Pseudomonas spp. thrives. That they found the optimal concentrations of phosphates (650–1250 mg/l), ammonia chloride (10–50 mg/l), and sulfates (10–20 mg/l) were amended into the similar aquifer.

Daniel Delille et al. [30] assessed the effects of nutrient and temperature on biodegradation of petroleum hydrocarbons in sub-Antarctic coastal seawater. They provided strong evidence of the presence of indigenous hydrocarbon degrading bacteria in Antarctic seawater and their high potential for hydrocarbon bioremediation. They studied three incubation temperatures (4, 10 and20°C) with two different concentrations of oil. Their research indicated that temperature had only a rather limited influence on petroleum degradation in the studied Antarctic seawater, especially when considering bioremediation as an efficient mean to remedy contaminated soils there. Also, the rate of oil degradation could be increased by the addition of a commercial fertilizer to a larger extent than elevated seawater temperature and global warming should not significantly increase oil biodegradation in Antarctica waters in the future.

Liang-Ming Whang et al. [31] investigated the effects of pH and ammonium concentrations on the potential application of two biosurfactants, surfactin (SF) and rhamnolipid (RL), and enhancing diesel biodegradation with a series of bench-scale

experiments and Compared to the experiments without biosurfactant addition. They found that an optimum pH condition for microbial growth and diesel biodegradation occurs at pH 7.2. Decreasing pH to 5.2 or increasing it to 8.4reduces those parameters considerably. Further increase of pH to 8.4, however, does not seem to negatively influence biodegradation and biomass growth. They also found that, an optimum ammonium addition for microbial growth and diesel biodegradation had found between (200 - 300mg-N/L), but a dramatic decrease in growth and biodegradation occurs at ammonium addition up to 450 mg-N/L.

Korda et al. [32] studied the sampling and analytical methods, along with available microorganisms, which used in situ treatment sand commercial microorganisms currently used in petroleum hydrocarbon bioremediation. Each treatment method is briefly described and its advantages and limitations. They demonstrated that certain bacterial strains have inability to break down or transform the chemical substances present in petroleum hydrocarbons. Oil-spill bioremediation technics aim to providing favorable conditions of oxygen, temperature and nutrients to maximize biological hydrocarbon breakdown. Such methods have been applied successfully in restoring polluted seashores, airports, military operations, power plants, etc. They focused on the commonest petroleum hydrocarbon bioremediation methods currently used. Emphasis has been
Placed on sampling and analytical methodologies, microorganisms, and treatment enhancement by supplying nutrients, oxygen, etc.

Jeong Myeong Kim et al. [39] designed a bacterium strain B113, able to degrade benzene, toluene, and ethylbenzene compounds (BTE). They had isolated that strain from

gasoline-contaminated sediment at a gas station in Geoje; Korea.it was belonged to the genus of Acinetobacter based on 16S rRNA gene sequences. The rate of biodegradation were relatively low in MSB broth, but the addition of yeast extract had an important impact on the biodegradation of BTE compounds, they had suggested that yeast extract was necessary factor for its growth or BTE biodegradation activity. Also, they noticed that biodegradation of BTE compounds occurred very quickly in slurry systems amended with sterile soil. Furthermore, they had found if soil was combusted to remove organic matters, the enhancement factor on biodegradation was lost.

J G Leahy and R R Colwell [34] reviewed The ecology of hydrocarbons degradation by microbial population sin, they were emphasized on the physical, chemical, and biological factors which contribute the hydrocarbon biodegradation, Also, they had showed that rates of biodegradation depend on the composition, state, and concentration of the hydrocarbons. Furthermore, Temperature, oxygen and nutrient levels are important in terrestrial ecosystems. While, salinity and pressure may affect on biodegradation rates in some aquatic ecosystems. Also, moisture and pH may limit biodegradation rates in soils. With respect to exposing of microbial communities to hydrocarbons which will increases the rate of hydrocarbon degradation.

Marion Børresen et al. [35] assessed the biodegradation potential of hydrocarbons in contaminated soil from a permafrost site. They showed that Biodegradation of hydrocarbons in the Arctic and Antarctic is very slow, due to low temperatures (they had used 5°C in the lab as temperature degree), with low contents of nutrients, organic matter and water. They designed the experiments as liquid cultures and soil microcosms, the

liquid cultures had shown that 18–54% of the initial diesel concentration was degraded after 32 days, while, the soil microcosm experiments had shown 0.9–15.8% of the initial hexadecane concentration was degraded after 128 days. Furthermore, they provided information regarding the indigenous microorganisms' capability to degrade different compounds of hydrocarbons under optimized conditions.

Abdel-Alim H. El-Sayed et al, [36] isolated two types of microorganisms from oil contaminated soil samples which were gathered from coastal area of Saudi Arabia on the Arabian Gulf Able to Degrade Arab Crude Oil. One of them *(pseudomonas sp.)* had proved its ability to make degradation to Arab crude oil added to salts solution, sterile and no sterile Gulf water. But, the other failed to adapt itself in salts solution. About 80% of Arab crude oil added had disappeared within 10 days of incubation by Pseudomonas sp..Also, the optimum degradation percent was at temperature of 25°C and 2.5 mg/ml Arab crude oil concentration. Adding extra nutrients had activated the degradation process and phosphorus had given the best rate of biodegradation among other nutrients. Also, increasing the amount of bacteria in the presence of sufficient nutrients had affected directly to the biodegradation rate.

Albert L. et al. [37] studied the biodegradation of benzo & pyrene (BaP) as high molecular weight polycyclic aromatic hydrocarbons (compounds containing four or more fused benzene rings). There is concern about the presence of (BaP) in the environment and carcinogenicity, teratogenicity and toxicity. (BaP) could inter inside food chain and cause human exposure through food consumption. They studied the ability of bacteria, fungi and algae to degrade high molecular weight polycyclic aromatic hydrocarbons,

including pathways for (BaP) biodegradation, and how to improve microbial degradation of (BaP).

D.J.Lacotte et al. [38] improved the biotreatment of Arabian Light crude oil in synthetic sea water by a mixed culture of bacteria. The evaluated the effects of fish meal, meat meal 80 % proteins and sophorolipids (with concentration at 0, 5 mgml) on the biodegradation during a three-week incubation using GC/FID and GC/MS analysis of the hydrocarbons in remaining oil. They had involved no nitrogen and phosphorous limits when used them as source of nutrients. Biodegradation processes in the first four days of the incubation supplemented with sophorolipids are accelerated two fold over controls. They demonstrated the natural limitation of the microbial oxidation of crude oi1 such as the limiting conditions in nitrogen and phosphorous assailable by microorganisms. Also, they had assessed the potential usefulness of bioremediation using N and P fertilizers to cleanup coastal environment contaminated with petroleum hydrocarbons.

J T Dibble and R Bartha [39] studied, evaluated and optimized the environmental parameters which affect on biodegradation in soil of oily sludges. The parameters which studied were soil moisture, pH, mineral nutrients, micronutrients, organic supplements, Treatment rate, teratment frequency, and incubation temperature. Oil sludge biodegradation was optimal at a soil water-holding capacity of 30 to 90%, a pH of 7.5 to 7.8, C:N and C:P ratios of 60:1 and 800:1, respectively, and a temperature of 20 ^{o}C degree and above.

Fernando Rojo [40] studied Alkanes compounds which are saturated hydrocarbons, very inert and most of them can be efficiently degraded by several microorganisms. He focused on the biochemical pathways and how the expression of pathway genes is regulated and integrated within cell physiology. Also, he showed that Alkanes can constitute up to 50% of crude oil, and also produced by many living organisms such as plants, green algae, bacteria or animals. However, he found that several microorganisms, both aerobic and anaerobic, can use diverse alkanes as a source of carbon and energy.

K. S. M. Rahman et al. [41] assessed the optimum conditions for biodegradation of Bombay High BH crude oil. They isolated 130 oil degrading bacterial cultures from contaminated soil samples, but they made a consortium of (Micrococcus sp. GS2-22, Corynebacterium sp. GS5-66, Flavobacterium sp. DS5-73, Bacillus sp. DS6-86 and Pseudomonas sp. DS10-129) for studying the efficiency of crude oil biodegradation. They noticed that using Individual bacterial cultures had showed less growth and biodegradation than the bacterial consortium. They showed that at 1% crude oil concentration, the consortium degraded 78% of BH crude oil, followed by 66% by Pseudomonas sp. DS10-129, 59% by Bacillus sp. DS6-86, 49% by Micrococcus sp. GS2-22, 43% by Corynebacterium sp. GS5-66 and 41% by Flavobacterium sp. DS5-73. Futthermore, The rate of biodegradation by bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1% to 10%. Also, they had found that the optimum Temperature was at 30 °C and the optimum pH was at 7.5 for maximum biodegradation.

This study used a consortia of two types of bacteria (*Pseudoxanthomonas* sp. and *Bacillus* sp.) as indigenous bacteria which isolated from contaminated sandy soil with petroleum hydrocarbons, and assessed the effects of three environmental factors on removal of total petroleum hydrocarbons (TPH), they were (nutrition , pH and temperature) at three degrees for each.

CHAPTER THREE:

MATERIALS AND METHODS

3.1. COLLECTION OF SOIL SAMPLES:

Contaminated sandy soil with oil samples were collected from already polluted sites with area of (3 – 30 m^2) from Saudi Aramco oil field in Jaddah, western region of Saudi Arabia.

The samples were collected by removing 2 inches depth from ground surface then 100 gm were collected from the third inch depth (randomly from different spots, through using a sterile scope (spatula). Samples were kept in sterilized plastic bags and transported to the laboratory for further analysis.

3.2. PREPARATION OF SOIL SAMPLES:

One thousand gram of soil was kept in the oven at 120°C for 24 hours for the following purposes:

- Killing all organisms which contaminate the samples.
- Air drying the soil samples.
- Removing volatile portion of hydrocarbon from the soil.

Soil samples were grounded and homogenized by sieving them through sterile sieve number 0, 0197 inch mesh to remove soil debris. Samples were stored inside a closed

Container and kept in the refrigerator at 4 °C until needed. One thousand gm of soil were kept in the refrigerator. These samples were used for the isolation of indigenous microorganisms' capable of degrading crude oil.

3.3. PREPARATION OF CULTURE MEDIA:

The mineral salt medium (MSM) was modified from Weissenfels et al. [42]. The composition of the medium was: 1.0g K_2HPO_4, 1.0g NH_4NO_3, 0.2 g $mgSO_4.7H_2O$, 0.1 g $CaC_{l2}.2H_2O$, 0.1 g NaCl, 0.01g $FeCl_3.6H_2O$, 3 ml trace element solution and deionized water to bring the volume up to 1 liter. MSM media was autoclaved at 120°C and 3 bar for one hour. The final pH was adjusted to 7± 0.2 by using 0.1 M HCl or 0.1 M NaOH.

3.4. BACTERIA ENRICHMENT:

One hundred grams of contaminated soil plus 200 ml of autoclaved water were added to 500 ml Erlenmeyer flask [Figure 1], the flask was kept in the shaker incubator at 30°C [Figure 2]. The supernatant was taken after 24 hours and added to five replicates of 100 ml nutrient broth in 250 ml Erlenmeyer flasks. The flasks were incubated using shaker incubator at 150 rpm and 30 °C for 3 days. After three days of enrichment, aliquots from each flask were spread on 0.5% crude oil agar plate as well as Nutrient agar plates. Crude oil plates were incubated at 30 °C for 10 days, Nutrients agar plates were checked for growth after 48-72 hours [Figure 3].

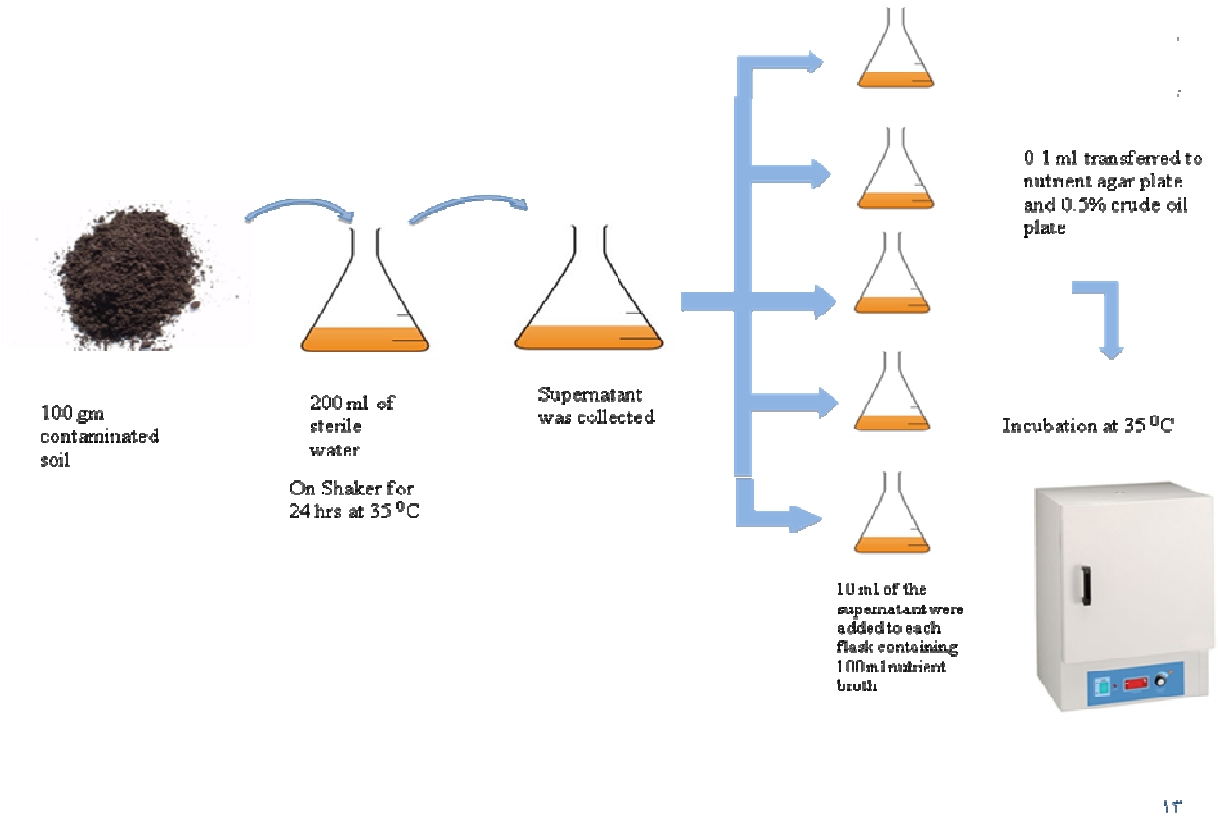

FIGURE 1: BACTERIA ENRICHMENT

FIGURE 2: WATER BATH SHAKER INCUBATOR

FIGURE 3: INCUBATOR

3.5. BACTERIAL COUNTS:

Aliquots of the cultured bacteria were inoculated into fresh nutrient broth medium and incubated in the same conditions to an absorbance at 600 nm of 0.50 ± 0.025. Cells were harvested by centrifugation at 4000 rpm for 10 min at 4O°C [figure 4], washed twice with a sterile 0.9% NaCl solution. Samples were diluted by serial dilutions to 10^{-10}. Bacteria were analyzed by plating of 0.1 ml from each dilution on nutrient agar plates (on duplicates). Colonies were counted after incubation at 37°C for 24 h [43, 44].

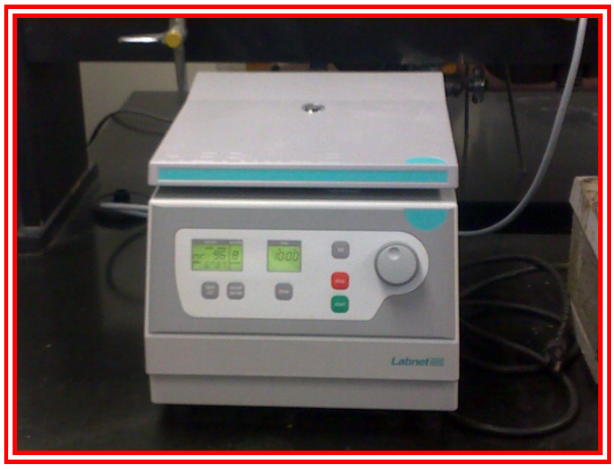

FIGURE 4: CENTRIFUGE

3.6. BACTERIAL IDENTIFICATION by 16S rRNA SEQUENCE:

3.6.1. DNA Extraction

Bacterial genomic DNA was extracted from the 2 isolates using NORGEN BIOTEK bacterial genomic DNA isolation kit.

3.6.2. PCR amplification of 16S rRNA genes

The amplification was conducted with universal primers (Thermo) designed to anneal to the conserved regions of bacterial 16S rRNA genes. The properties of these primers are shown in table 1.

Table 1. Primers used in the 16S rRNA PCR analysis

Primer name	Primer sequence 5'-3'	Primer size	Tm (°C)	GC %	Seq. amplified
8-27 F	5'-AGA GTT TGA TCC TGG CTC AG-3'	20	61.5	50.0	The whole gene
1492 R	5'-GGT TAC CTT GTT ACG ACT T-3'	19	53.7	42.1	The whole gene

The PCR reaction contain 10 µL 5x Phusion$_{TM}$ GC buffer (contains 7.5 mM $MgCl_2$, which provides 1.5 mM Mg Cl_2 in the final reaction conditions) (Finnzymes, Finland), 0.5 µL dimethyslufoxide (DMSO) (aids in the denaturing of templates with high GC content) (Finnzymes, Finland), 5 µL of 2 mM of deoxyribonucleotides triphosphates mix, (BioLabs, UK) (provides 0.2 mM of each deoxyribouncleotide triphosphate in the final reaction conditions), 0.25 µL of each primer (provides 0.5 µM of each in the final reaction conditions), 0.5 µL of Phusion$_{TM}$ DNA polymerase (provides 0.02 U/µL) (Finnzymes, Finland), and 2 µL of DNA template with a final concentration ranging between 15-30 ng in a total volume of 50 µL. Amplification was performed with the thermocycler (WhatmanBiometra, Germany) PCR reactions were heated to 98 °C for 4 minutes to achieve the initial DNA denaturation, followed by 30 cycles with the

following cycling profile: 98 °C for 30 seconds; for denaturation, 53 °C for 30 seconds; for annealing, and 72 °C for 1 minutes; for extension. A final extension was carried out after the amplification reaction at 72 °C for 10 minutes.

3.6.3. Detection of the PCR products

PCR products, stained with 6x orange loading dye (fermentas), were electrophoresed through a 1.2%(w/v) agarose gel (Saveen Werner, Sweden), in 1X TAE buffer, stained with ethidium bromide (Mercury, USA). 10 µL of 1Kb DNA marker (O'gene ruler; Fermentas) was loaded to detect the 16S rRNA bands which were then visualized by UV transillumination. The amplified 16S rRNA bands were cut and stored at -20 °C.

3.6.4. Purification of PCR products

The PCR products were purified using QIAEX® II Gel extraction Kit (150) (QIAGEN). This was followed by measuring the concentration of the purified DNA using the NanoDrop (NanoDrop Technologies, INC. Wilmington, USA, NanoDrop® ND-1000; full-spectrum U.V/risspectrophotometer). A concentration of 20 ng/100 base (i.e., 300 ng of the 1.5 kb 16S DNA) is required for sequencing.

3.6.5. Sequencing

In order to be sequenced, the purified DNA samples were dried using the freeze-dryer (Labconco Lyph-Lock, model 7750, AbNinolab, and Stockholm, Sweden). Samples were

labeled and kept in the refrigerator until they were sent for sequencing. Sequencing was carried out by Biosciences group at KAUST.

3.7. EXPERIMENTAL SETTING:

Experimental setting was conducted based on the method described by Ajay Singh, et al. [45] and Abdel-Alim H. El-Sayed [36] with some modifications.

Experiments were conducted using 125 ml Erlenmeyer flasks. Flasks were sterilized by autoclaving at 121 °C for 15 minutes. Forty ml of mineral salts medium was added to each flask. All flasks were kept in shaker incubator (at 120 rpm). The mixture of 2 bacterial isolates was added to these flasks to a final cell density of 1.2×10^8 cfu /ml (2ml was added to each flask). Experiments were carried out for the three environmental factors and devoted to study the temperature, pH and nutrition as factors affecting biodegradation.

Three sets of flasks (six flasks for each set) were kept in water-bath shakers at (25°C, 35°C and 45°C). The media in the flasks was adjusted to pH 7± o.2. The experiments were conducted for 20 days; aliquots from each flask were taken every 4 days for analysis. Control flasks were kept along with the experimental flask at all times. These control flask were, one flask with contaminated soil but without bacteria which kept running for 20 days (to ensure that no biodegradation will be carried out without bacteria), and the second flask was contaminated soil with the media which used directly to analyse the TPH at the begging of the experiment (0- time).

The same steps used above were repeated to study the effect of pH by conducting the experiment at PHS, 5, 7, and 9 at 35 ^{0}C.

The effect of Ammonium Chloride, Sodium Nitrate and Urea (10 mM) were also investigated using the same method above in order to determine the best and most appropriate nitrogen source for conducting its effect at different concentrations on the biodegradation ability.

Ammonium chloride was chosen to study the effect of nitrogen on the biodegradation ability of crude oil. The concentrations used were 5, 10, and 20 mM.

3.8. **ANALYSIS OF TPH BY GRAVIMETRIC METHOD:**

The gravimetric method described by Mario Villalobos et al. [46] was used for analysis of TPH (total petroleum of hydrocarbons).

Soil samples were sieved and dried at 105 $^{\circ}$C for 12 h. These were homogenized mechanically for several hours, and approximate accurately weighed 10 g subsamples were placed in round flasks previously dried (105°C) to constant weight. Ten grams of anhydrous Na_2SO_4 were added to create a free flowing powder, and 35 ml n-hexane was used for extraction in an ultrasound bath [figure 5], the conditions of which were investigated for optimal extraction efficiency. The extracts were filtered through a column packed first with 0.6 g treated cotton or glass fiber with 5 g treated silica gel, and 1 g treated celite, respectively, and washed down with additional 25 ml hexane to

Complete 60 ml in the final liquid extract for analysis [figure 6]. The hexane was evaporated in a rotary evaporator, followed by drying of the flask outer walls with lint-free absorbent paper and evaporation of the remnant hexane under variable conditions. The residue was weighed in an analytical balance and designated as TPH [figure 7]. [47-49]

Figure 5: Ultra-Sonic Bath

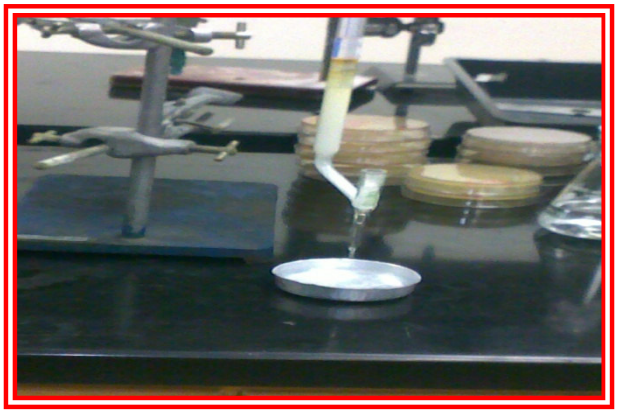

Figure 6: Gravimetric Method

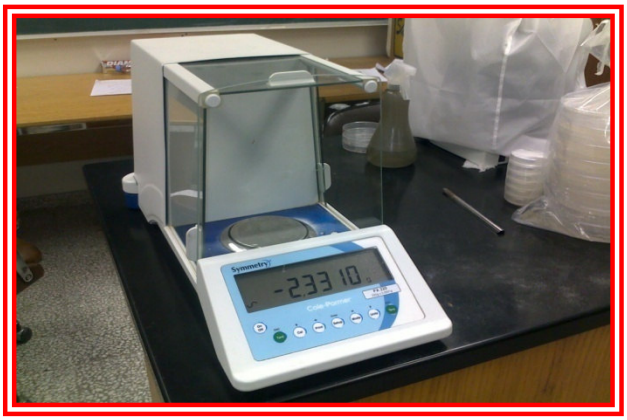

Figure 7: Digital Balance

CHAPTER FOUR

RESULTS AND DISCUSSIONS

The main advantages of bioremediation processes are their relatively low cost, process flexibility, benign nature environmentally, and on-site utility [45].

Many factors such as: soil moisture, pH, temperature, aeration, nutrients, type of soil, type of contaminants… affect the ecology of the microbial population and biodegradation of hydrocarbons [45].

For succession bioremediation treatment process of soil, it is important to consider number of factors such as: type and extent of contamination (in this thesis, TPH inside each sample was around 0.19 grams),Present of bacteria(in this thesis 2.4 X 10^8 cfu /mL had used to each sample), duration since contamination (in this thesis it was 3 weeks), optimal microbiological conditions (specially environmental factors such as nutrients, concentration of nutrients, pH and temperature) soil characteristics, proper bioremediation technique (biodegradation by bacterial activities), and appropriate analytical method(such as gravimetric method or GC). All above criteria are very important to minimize risk of failure in terms of effort, time, and money [45].

The optimum contaminant loading level for biotreatment is about 5% (by weight) of oil[45],in this study the amount of TPH inside each 10 grams was close to 0.2 gram, that means the amount of TPH represent 4% of dry soil, namely the amount of TPH was at

Optimum level. Maximum degradation rates are typically observed in the upper 10–15 cm of the soil surface [45] (as it had happened in this study).

Even with optimum conditions the biodegradation process will still incomplete. The residual hydrocarbons may be acceptable if it have no significant impact on ecosystem and do not have a risk on groundwater resources. [45]

The main objectives of this study were to isolate and characterize bacterial isolates capable of degrading crude oil and to investigate the potential of enhanced in situ biodegradation treatment of petroleum hydrocarbon in contaminated soils.
The potential of the isolated strains to degrade crude oil were investigated under three environmental factors: pH, temperature, nutrition with different concentrations of nutrition.

4.1. ENRICHMENT OF BACTERIAL ISOLATES:

Soil is a complex medium, dynamic and living habitat for organisms specially bacteria because of their abundance, their diversity and their metabolic activities. Bacteria also reflect the past history of a given environment. For that it is important to understand the structure and diversity of soil bacterial communities and their responding to various natural or man-made interactions [50].

However the samples which they had analyzed in this thesis were rich of bacteria, Enrichment of bacterial cultures from contaminated soil were carried out to determine number of bacteria to be used as inoculums or as starter cells for our experiments.

The number of bacterial was determined to be around 1.2 X 10^8 cfu /ML. In this study 2 ML. were used for each sample. The addition of bacteria (inoculums) had made to accelerate and make a higher effective to biodegradation process [51].

Regular additions of active microbial culture (such as bacteria) will maintain a constant rate of biodegradation of toxic substances (hydrocarbons) in case of high death rates of microorganisms during biodegradation process [51]. Microorganisms suitable for the biotreatment of hazardous substances can also be isolated from the natural environment [51].

On the other side, aggregation of microbial cultures may because problems associated with limited of diffusion (slow diffusion of nutrients and the metabolism of them [51].

4.2. BACTERIAL IDENTIFICATION BY 16S rRNA SEQUENCE:

Two bacterial strains were isolated following the enrichment procedures mentioned in the materials and methods above. These tow starins were chacracterized by sequencing of the 16S rRNA gene.

The 16S rRNA anlaysis for the 2 strains used in this study were indicated that these 2 strains are *Pseudoxanthomonas* sp. [Figure 8] and *Bacillus* sp. [figure 9] the sequence for these stains as follows:

4.2.1. Pseudoxanthomonas sp. [Figure 8]

TCCTTGCGGTTAGCTACCTGCTTCTGGTGCAACCAACTCCCATG

GTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCG

CAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGGA

GTCGAGTTGCAGACTCCAATCCGGACTGAGATGGGGTTTCTGG

GATTGGCTCCCCCTCGCGGGTTCGCAGCCCTCTGTCCCCACCAT

TGTAGTACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTG

ACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTT

AGAGTTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGTTG

CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG

ACGACAGCCATGCAGCACCTGTCTCGCGGCTCCCGAAGGCACC

CGCCCATCTCTGGGCAGTTCCGCGGATGTCAAGACCAGGTAAG

GTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTG

TGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGT

ACTCCCCAGGCGGCGAACTTAACGCGTTAGCTTCGATACTGAGT

CCCAAATTGAACCCAACATCCAGTTCGCATCGTTTAGGGCGTGG

ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGC

CTCAGTGTCAGTGTTGGCCCAGGTGGCCGCCTTCGCCACGGGTG

TTCCTCCCGATCTCTACGCATTTCACTGCTACACCGGGAATTCC

GCCACCCTCTACCACACTCTAGTGACCCAGTATCCACTGCAATT

CCCAGGTTGAGCCCAGGGCTTTCACAACGGACTTAAGCCACCA

CCTACGCACGCTTTACGCCCAGTAATTCCGAGTAACGCTTGCAC

CCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGG

Figure 8 : Pseudoxanthomonas Sp. ,Gram Negative, Spherical In Shape

4.2.2. Bacillus sp. [figure 9]

TACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGG
GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCT
GATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGC
AGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTA
GCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGT
GTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCC
CACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCA
ACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATG
CACCACCTGTCACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAG
GGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGC
TTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTC
AATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAAGCGGA
GTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCT
AACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATC
TAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTA
CAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTC
TACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTG
CACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCG
GGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTA
C

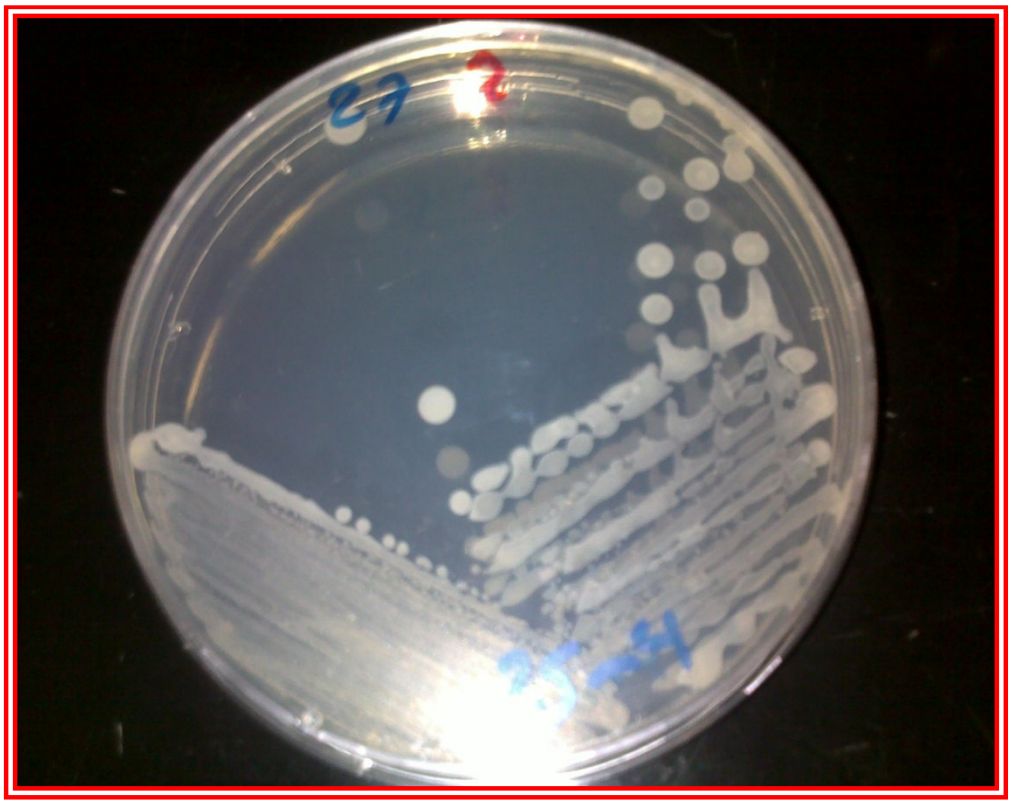

Figure 9 : *Bacillus sp.* ,Gram positive bacilli

4.3. DEGRADATION OF HYDROCARBONS:

RELIABLE EXPERIEMENT:

In order to investigate how much TPH in the contaminated soil sample, 3 samples (10 gram each) were analyzed for TPH by gravimetric method. The results showed that these 10 gm of contaminated soil contains about 0.2 gm of TPH.

4.3.1. EFFECT OF NUTRIENTS:

Bacteria that are both aerobic and heterotrophic are the most important in the biodegradation process. Bacteria require organic and inorganic nutrients such as nitrogen, phosphorus, magnesium; calcium, iron, and trace metals to support cell growth sustain biodegradation processes and maintain active bacterial populations. [45] In addition, cell death may occur in zones that do not receive sufficient nutrition or that contain inhibitory metabolites [51].

Nitrogen is the most important of these elements, as it is used in the proteins synthesis, structure of nucleic acids and cellular components. Elemental nitrogen present as gas in atmospheric and almost inert, for that, gaseous nitrogen must be “fixed” by bacteria in soil. [52] Also, Addition of nutrients to soil, such as nitrogen fertilizers, has been proven to enhance biodegradation of poly aromatic hydrocarbons (PAHs) [53].

Selection of the acceptor in biodegradation process depends on economical and environmental reasons. Nitrate is often proposed for bioremediation that it can be used by

Many microorganisms as an electron acceptor [51]. Ammonium is the best source of nitrogen [51] and the Urea is famous source of nitrogen. Thus, three kinds of nitrogen sources have been chosen to investigate their efficiency on biodegradation process: Ammonium Chloride (A.C), sodium nitrate (S.N) and Urea (U).

Ten mM of Ammonium Chloride, sodium nitrate and Urea were used to investigate their effect on the removal of TPH from the contaminated soil samples at 35 ^{o}C. [Figure 10] below illustrate the effect of these different nitrogen sources on the removal of TPH.

The removal of TPH were at higher rate of 10 mM Ammonium chloride (going down from 0.1846=100% to 0.0822 gm=44.5%), followed by 10 mM urea (going down from 0.1846=100% to 0.0888 gm=48%) and then 10 mM sodium nitrate (going down from 0.1846=100% to 0.0916 gm=49.5%). The removal rate was not significant as per the results in Figure 1 below.

The biodegradation rates of hydrocarbons under the three types of nutrients were very close from each other. The faster rate of biodegradation was noticed to be in the first four days (especially with ammonium chloride). Then in second four days, after that, the biodegradation rate become slower, and very slow in the last 4 days. This indicate that the activity of the bacteria and assimilation process was at higher rate in the beginning, (the oxygen rate is still high), decreasing of oxygen level will decreased the growth rate and enrichment of bacteria which will decrease the diffusion of oxygen. While this study had designed under aerobic conditions, and it is known the importance of O2 to biodegradation process.

These results in agreement with previous study about in vitro biodegradation of Arabian Light 250 by a marine mixed culture using fertilizers as Nitrogen and Phosphorous sources [38] which showed that biodegradation processes in the first four days of the incubation supplemented with sophorolipids are accelerated two fold over controls.

Based on the above results, Ammonium chloride was chosen to be used for studying the effect of nutrient levels.

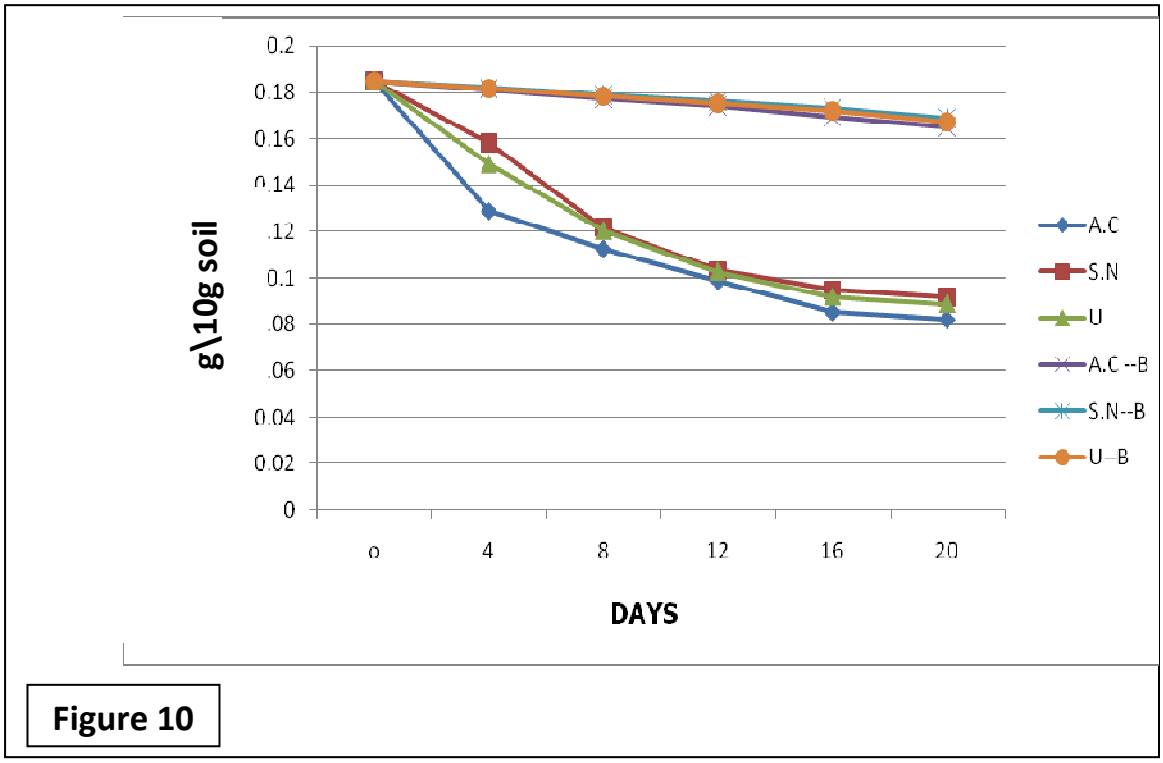

Figure 10

Figure 10: The effect of 10 mM of Ammonium Chloride (A.C), sodium nitrate (S.N) and Urea (U) on the removal of TPH from the contaminated soil samples at 35 ^{o}C.

Table 2- A: The effect of using of Ammonium Chloride(A.C), sodium nitrate(S.N) and Urea(U) on biodegradation rate (by grams & % 0f TPH)

Time days	A.C	S.N	U
0	0.1846= 100%	0.1846= 100%	0.1846= 100%
4	0.1287= 70%	0.1581= 85.5%	0.149= 80.5%
8	0.1124= 61%	0.1216= 66%	0.1203= 65%
12	0.0987= 53.5%	0.1031= 56%	0.1027= 55%
16	0.0853= 46%	0.0946= 51%	0.0919= 49.5%
20	0.0822= 44.5%	0.0916= 49.5%	0.0888= 48%

Table 2- B: A comparison between the values of TPH for different nutrients at 0-time and the end-time (without treatment) (by grams & % 0f TPH)

Time days	A.C –B	S.N—B	U—B
0	0.1846=100%	0.1846=100%	0.1846=100%
20	0.165= 89.5%	0.1687= 91.5%	0.1672= 90.5%

4.3.2. EFFECT OF NUTRIENTS LEVELS:

Five, ten & twenty mM of Ammonium Chloride were used to investigate their effect on the removal of TPH from the contaminated soil samples at 35 ^{o}C.

[Figure 11] below illustrate the effect of these different concentrations on the removal of TPH.

The removal of TPH was the highest at 5 mM Ammonium chloride (going down from 0.1912 gm=100% to 0.0573= 30%), followed by 10 mM Ammonium chloride (going down from 0.1912 gm=100% to 0.0916= 48%) and then 20 mM Ammonium chloride (going down from 0.1912 gm=100% to 0.1479= 77.5%).

The biodegradation rates with the three concentrations of nutrients were varied from each other. The faster rate of biodegradation was noticed to be in the first four days (especially with 5 mM ammonium chloride). Then in second four days, the biodegradation rate became slower, and very slow in the last 4 days. This means that the activity and the assimilation of bacteria was at highest level in the beginning, which is in agreement with several studies [38], Due to enrichment of bacteria, the assimilation level will decrease; this indicates that the decreasing trend on biodegradation process will happen.

Based on the above results, 5 mM Ammonium chloride was chosen as an optimum concentration.

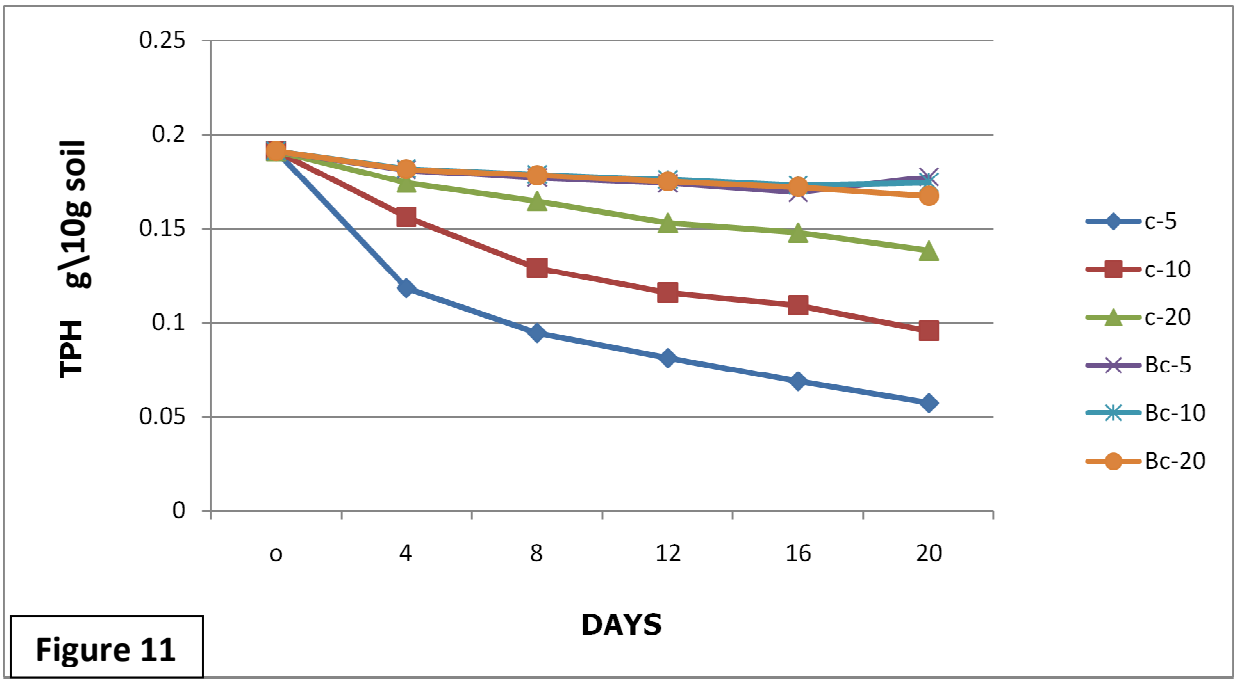

Figure 11: The effect of five, ten & twenty mM of Ammonium Chloride at pH=7 at 25 ^{o}C on the removal of TPH from the contaminated soil samples.

Table 3-A: Effect of 3 values of concentrations of Ammonium Chloride at pH=7 & temperature at 35 ^{o}C on the removal of TPH. (By grams & % 0f TPH)

Time days	c-5	c-10	c-20
O	0.1912= 100%	0.1912= 100%	0.1912= 100%
4	0.1283= 67%	0.1561= 81.5%	0.1743= 91%
8	0.0996= 52%	0.1289= 67.5%	0.1646= 86%
12	0.0813= 42.5%	0.1159= 60.5%	0.1571= 82%
16	0.0689= 36%	0.1041= 54.5%	0.1531= 80%
20	0.0573= 30%	0.0916= 48%	0.1479= 77.5%

Table 3-B: A comparison between the values of TPH at 0-time and the end-time (without treatment). (By grams & % 0f TPH)

Time days	C=5	C=10	C-20
0	0.1912= 100%	0.1912= 100%	0.1912= 100%
20	0.1773= 92.5%	0.1745= 91%	0.1672= 87.5%

However, excessive amounts of certain nutrients can repress microbial metabolism. Thus, providing an appropriate balance of nutrients will achieve high level of bacterial growth and thus accelerated rates of TPH biodegradation [45]. Excessive localised microbial growth in nutrient-injected areas, resulting in “biofouling” which consuming the oxygen [55]. Also, amendments to rectify nutrient deficiencies must be optimal, as too high amounts may lead to eutrophication which consume oxygen, and too little may result in suboptimal biodegradation. [56]

These results in agreement with a study about Application of rhamnolipid (RL) and surfactant for enhanced diesel biodegradation to investigate the Effects of ammonium concentration. It showed that an optimum amount of ammonium for microbial growth was found between 200 and 300 mg-N/L, while, a dramatic decrease in biodegradation and growth rate occurred at ammonium addition up to 450 mg-N/L [54].

All of this information interprets why the optimum concentration was at 5 mM, then, 10 mM which make more enrichment of bacterial growth which cause decreasing of oxygen diffusion, and that will lead to decreasing rate of biodegradation process but in little limitation. While, excessive amounts of nutrient as the concentration of 20 mM will suppress the biodegradation process to high extent as this result.

Also, Using an appropriate concentrations and ratios of nutrients will avoid limitation of bacterial growth or depletion of one essential nutrient while all other nutrients may be present in excess [45].

It is useful to mention the recommended ratio in the literature of carbon to nitrogen (Optimum C:N ratio), it is (100:1) Thus, to biodegrade 100 kg of petroleum hydrocarbons, 1 kg of nitrogen must be added as nutrient fertilizers as KNO3, NH4NO3, NH4Cl or Urea.[45]

In the field application, the use of gaseous formulations of nutrients had demonstrated to be better in distribution of nutrients rather than injection of nutrients, nutrient supplementing. [45, 57]

Sometimes it is difficult to extrapolate the results directly from the laboratory to the field. Nevertheless, successful bench- or pilot-scale test results are mostly useful in designing the full-scale bioprocessing system for bioremediation of hydrocarbon-contaminated soil. [58]

4.3.3. **EFFECT OF pH:**

The effect of pH on the removal of TPH was investigated. The media for bacterial growth on the TPH contaminated soil was adjusted to pH 5, 7 and 9.

[Figure 12] illustrate the effect of pH on the removal of TPH from the tested contaminated soil. The highest TPH removal rate was at pH 7.0 decreased from 0.1823gm=100% to 0.1009gm=55% after 20 days of incubation. At pH 5 and 9 the rate of TPH removal decreased and it was 0.1453= 79.5%

And 0.1478= 81% respectively. Table 3 summarizes the removal of TPH at the 3 different pHs.

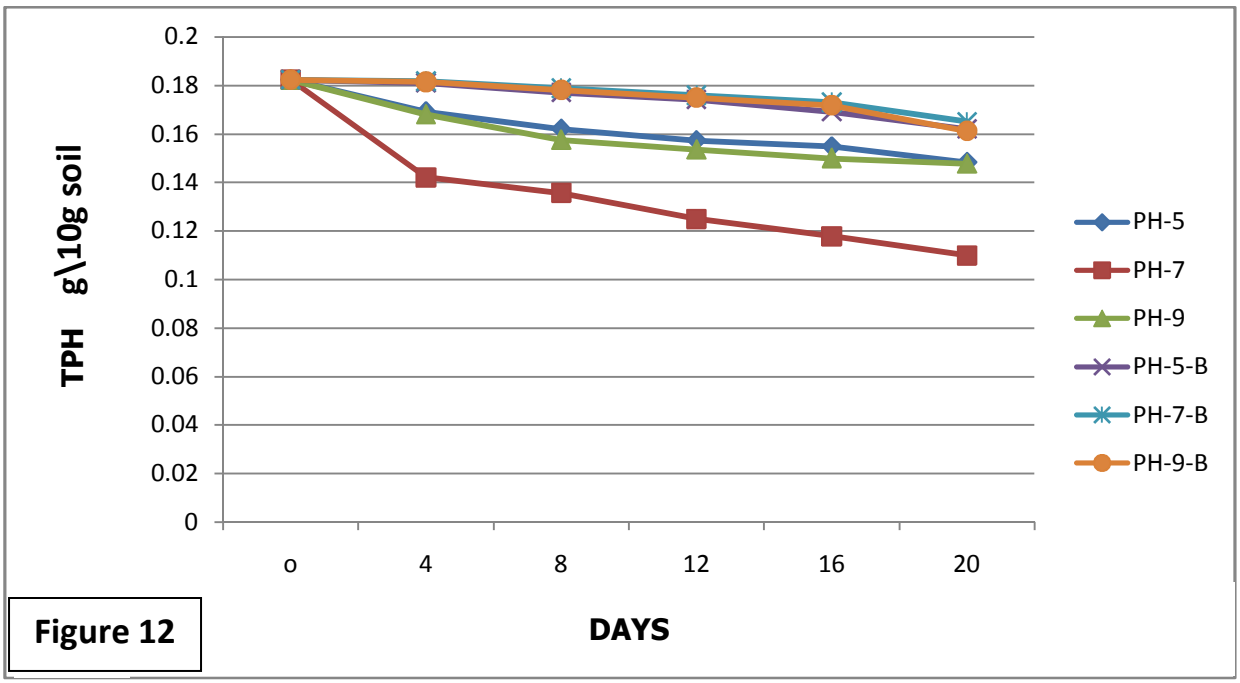

Figure 12: The effect of 10 mM of Ammonium Chloride at different values of pHs on the removal of TPH from the contaminated soil samples at 25 ^{o}C.

Table 4-A: The effect of using different pHs with 10mM Ammonium Chloride on biodegradation rate. (By grams & % 0f TPH).

Time days	PH-5	PH-7	PH-9
o	0.1823=100%	0.1823=100%	0.1823= 100%
4	0.1692= 93%	0.1421= 78%	0.1681= 92%
8	0.1621= 89%	0.1356= 74.5%	0.1576= 86%
12	*0.1573= 86%*	0.1249= 68.5%	0.1536= 84%
16	0.1518= 83%	0.1177= 64.5%	0.15= 82%
20	0.1453= 79.5%	0.1009= 55%	0.1478= 81%

Table 4- B: A comparison between the values of TPH for different values of pH at 0-time and the end-time (without treatment) (by grams & % 0f TPH)

Time days	PH-5-B	PH-7-B	PH-9-B
0	0.1823=100%	0.1823=100%	0.1823=100%
20	0.162= 90%	0.1651= 90.5%	0.1612= 88.5%

These results are harmonious with the knowledge that the optimum pH values for biodegradations of hydrocarbons in soil ranges from (6 to 8)[45] while the growth rate in most microbes and bacteria in general is efficiently within the pH range from 5 to 9 [51].

These results in agreement with a study about Application of rhamnolipid (RL) and surfactant for enhanced diesel biodegradation to investigate the Effects of pH. It showed that an optimum pH condition for microbial growth and diesel biodegradation was found to be at a pH 7.2, while decreasing pH to 5.2 or increasing it to 8.4 reduced those parameters considerably. [54]

Species that have adapted to grow at pH values among (5-8) called neutrophils, Species that have adapted to grow at pH values ($pH < 4$) are called acidophiles and Species that have adapted to grow at pH values ($pH > 9$) are called alkaliphiles. [51]

Thus, the pH of the medium must be maintained at optimal values for high efficiency biodegradation by certain physiological groups of microorganisms. The optimum pH value may be maintained physiologically by the addition of a pH buffer or pH regulator in the assimilation of ammonium, nitrate, or ammonium nitrate, leading to decreased pH, increased pH, or neutral pH, respectively [51]

In case of application in the field and in case of acidic soil ($pH < 6$), lime or calcium carbonate may be added to increase the pH value. For alkaline soil ($pH > 8$) sulfur, ammonium, sulfate, or aluminum sulfate may be added to lower the pH value. [45]

Based on the above results, Ammonium chloride was chosen at pH= 7 as the optimum value for biodegradation process and to be used for the studying of the effect of temperature degrees.

4.3.4. EFFECT OF TEMPERATURE:

The effect of temperature one of the very important factors on the growth of microorganisam as well as the biodegradtion of crude oil, the active range of temperature is very wide, because it depends on several factors specially the adaptation of indigenous microorganisms with their environment. Removal of TPH was investigated at 3 different temperatures (25, 35, and 45 ^{o}C).

[Figure 13] illustrate the effect of temperature on the removal of TPH from the contaminated soil by the isolated microorganisms. The rate of biodegradation (removal of TPH) was the highest at 35 ^{o}C which showed the reduction of TPH from 0.1929 gm=100% to 0.0859= 45% after 20 days of incubation, followed by the removal of TPH at 25 oC which showed the reduction from 0.1929 gm=100% to 0.1103= 57%. The lowest reduction rate was at 45 ^{o}C which reduced the TPH from 0.1929 gm =100% to 0.1387= 72%.

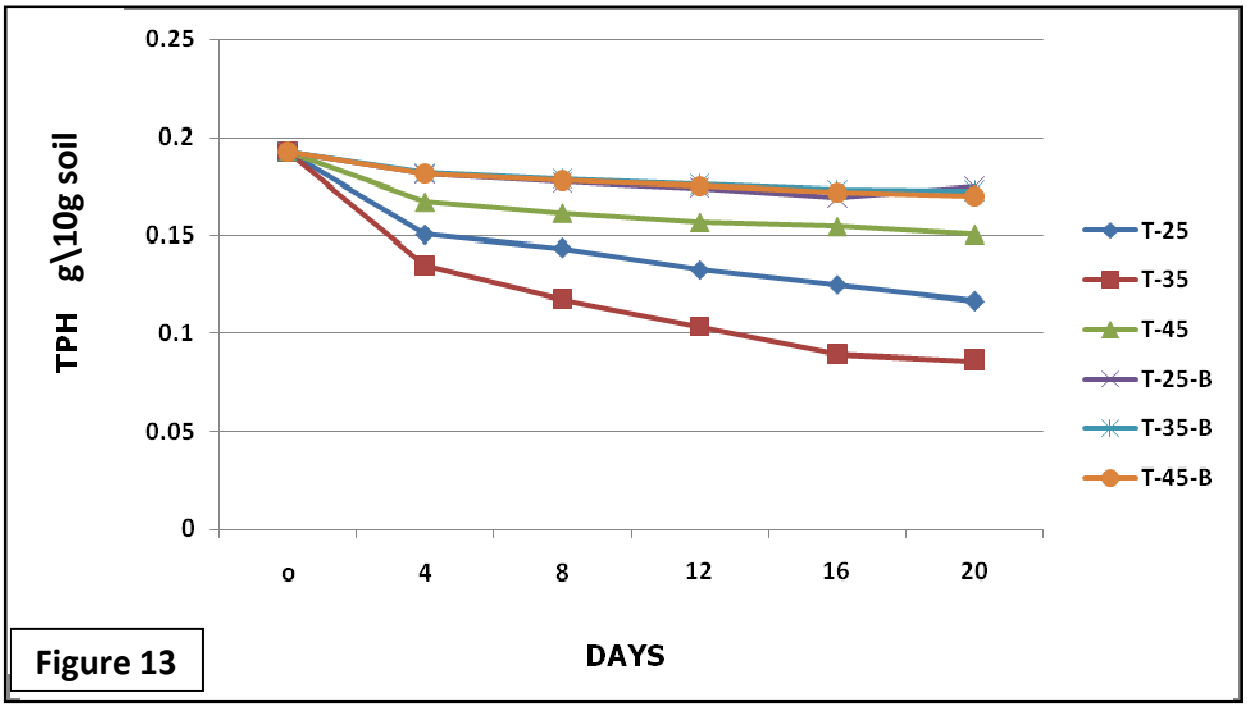

Figure 13: illustrate the effect of 10 mM of Ammonium Chloride at pH=7 with different temperatures on the removal of TPH from the contaminated soil samples.

Table 5-A: Effect of temperatures with ten mM of Ammonium Chloride at pH=7 on the removal of TPH. (By grams & % 0f TPH)

Time days	T-25	T-35	T-45
0	0.1929= 100%	0.1929= 100%	0.1929= 100%
4	0.1503= 78%	0.1345= 70%	0.167= 86%
8	0.1435= 74.5%	0.1174= 61%	0.1512= 78.5%
12	0.1322= 68.5%	0.1031= 53.5%	0.1463= 76%
16	0.1245= 64.5%	0.0891= 46%	0.1421= 73.5%
20	0.1103= 57%	0.0859= 45%	0.1387= 72%

Table 5-B: A comparison between the values of TPH at 0-time and the end-time (without treatment). (By grams & % 0f TPH)

Time days	T-25-B	T-35-B	T-45-B
0	0.1929= 100%	0.1929= 100%	0.1929= 100%
20	0.1747= 91%	0.1724= 89%	0.1699= 88%

These results are harmonious with the knowledge that optimum temperature for microbial biodegradation process range from 25 °C to 35 °C [45].in this study, the optimum biodegradation rate was at 35°C followed by the rate at 25°C, and this is due to adaptation of bacteria to high temperature degree.

Biodegradation rates are expected to be slow below 15 °C or above 40 °C [45] because the effect of low or high temperatures on activity of enzymes [5] and that is clear in this investigation at 45°C which give the lowest efficiency of biodegradation.

Also, there are two factors which they have an important role in biodegradation process related to temperature factor: moisture and evaporation.

The moisture content depends strongly on the type of soil (for example, clay soil and high organic matter soils retain higher moisture content than sandy soil). It is important to determine the amount of moisture for each sample to assess the biodegradation. The optimum moisture content for stimulating biodegradation of hydrocarbon ranges from (50 - 80%) of the moisture content at field capacity [45], for instance, if the soil moisture at field capacity was 20 g of water per 100 g of dry soil, the moisture of soil should be(10 - 16 g) of water per 100 g of dry soil. In this study, and to avoid the moisture factor, the weathering was made as mentioned in chapter of materials and methods to make sure that moisture content is fixed in each sample.

Also, it is important to consider the factor of evaporation, that (15 - 60%) of fuel hydrocarbons (diesel, jet fuel, and heating oil) can be lost during soil bioremediation due to evaporation [45] (Salanitro 2001). At room temperature (20 °C), most light hydrocarbons (carbon numbers up to C16) evaporate from soil directly when contact with air. Heavy hydrocarbons (> C16) are likely to volatilize in intense sunshine. These loss mechanisms during field or laboratory bioremediation studies should be considered [45], and to avoid this factor, the weathering had made as mentioned in chapter of materials and methods to volatile light hydrocarbons.

CHAPTER FIVE

CONCLUSION & RECOMMENDATIONS

5.1. CONCLUSION

Two bacterial strains were isolated from already contaminated soils; these two starins were chacracterized by sequencing of the 16S rRNA gene. They were: *Pseudoxanthomonas* sp. and *Bacillus* sp.The potential of the isolated strains to degrade crude oil were investigated under three environmental factors: pH, temperature and nutrition with different concentrations of nutrition. The efficiency of biodegradation on removal of TPH was good, which reach to 70% at optimum conditions which were with 5mM of ammonium chloride at pH=7 and temperature degree at 35^{o}C.

5.2. RECOMMENDATIONS

Further studies are needed in this area to address:

- Other parameters should also be considered in more details such as oxygen levels and moisture content.
- Optimum conditions of the bioremediation treatment process need to be identified.
- Developing genetically modified bacteria for crude oil biodegradation enhancement.

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VITAE

Mr. Feras Mohammad Rasheed Salamah was born in 1971. He is Jordanian. He enrolled in Al-Fateh University, Tripoli, Libya, and received a B.Sc. degree from the Faculty of Science (department of Biology) in 1993.

He enrolled in Jordanian University in 1996 with the program of Master in (Environmental Science and Management).

He joined King Fahd University for petroleum and minerals, KFUPM, Dhahran in 2006 as graduate student to achieve the master degree in environment program.

Contact Address:

SAUDI ARABIA
Eastern Region,Dhahran,Rabwa
Dhahran 31261
p.o.Box 5080
00966541810100
ferasmrs@hotmail.com